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VEGF-B Promotes Endocardium-Derived Coronary Vessel Development and Cardiac Regeneration

Running Title: *Räsänen et al.; VEGF-B in Coronary Development and Cardiac Repair*

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Abstract

Background: Recent discoveries have indicated that, in the developing heart, sinus venosus and endocardium provide major sources of endothelium for coronary vessel growth that supports the expanding myocardium. Here we set out to study the origin of the coronary vessels that develop in response to vascular endothelial growth factor B (VEGF-B) in the heart and the effect of VEGF-B on recovery from myocardial infarction.
Methods: We used mice and rats expressing a VEGF-B transgene, VEGF-B-gene-deleted mice and rats, Apelin (Apln)-CreERT^2 and Npr3-CreERT^2 recombinase-mediated genetic cell lineage tracing and viral vector-mediated VEGF-B gene transfer in adult mice. Left anterior descending coronary vessel ligation was performed and EdU-mediated proliferating cell cycle labeling, flow cytometry, histological, immunohistochemical, and biochemical methods, single-cell RNA sequencing and subsequent bioinformatic analysis, micro-computed tomography, and fluorescent and tracer-mediated vascular perfusion imaging analyses were used to study the development and function of the VEGF-B-induced vessels in the heart.
Results: We show that cardiomyocyte overexpression of VEGF-B in mice and rats during development promotes the growth of novel vessels that originate directly from the cardiac ventricles and maintain connection with the coronary vessels in subendocardial myocardium. In adult mice, endothelial proliferation induced by VEGF-B gene transfer was located predominantly in the subendocardial coronary vessels. Furthermore, VEGF-B gene transduction prior to or concomitantly with ligation of the left anterior descending coronary artery promoted endocardium-derived vessel development into the myocardium and improved cardiac tissue remodeling and cardiac function.
Conclusions: The myocardial VEGF-B transgene promotes the formation of endocardium-derived coronary vessels during development, endothelial proliferation in subendocardial myocardium in adult mice, and structural and functional rescue of cardiac tissue after myocardial infarction. VEGF-B could provide a new therapeutic strategy for cardiac neovascularization after coronary occlusion to rescue the most vulnerable myocardial tissue.

Key Words: VEGF, revascularization, myocardial ischemia, cardiac protection, gene therapy.

Non-standard Abbreviations and Acronyms
AAV - adeno-associated viral vector
αMHC - α-myosin heavy chain
Apln - apelin
CMC - cardiomyocyte
CVD - cardiovascular diseases
DALY - disability-adjusted life years
DiI - 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate
EC - endothelial cell
5-ethynyl-2'-deoxyuridine - EdU
EF - ejection fraction
i.p. - intraperitoneal
LAD - left anterior descending
MI - myocardial infarction
Npr3 - natriuretic peptide receptor 3

microCT - micro computed tomography
PFA - paraformaldehyde
PlGF - placenta growth factor
RT - room temperature
SV- sinus venosus
scRNA - single-cell RNA
TG - transgene
UMAP - uniform manifold approximation and projection
VEGF - vascular endothelial growth factor A
VEGF-B - vascular endothelial growth factor B
VEGFR-1 - vascular endothelial growth factor receptor 1

Clinical Perspective

What is new?

- VEGF-B promotes coronary vessel development from ventricular endocardium to subendocardial myocardium, and improves reperfusion and cardiac function after myocardial infarction.
- In ischemic heart disease, this could provide a new possibility to improve cardiac perfusion.

What are the clinical implications?

- Regardless of rapid reperfusion of the ischemic or infarcted heart, insufficient subendocardial vessel perfusion ("no-flow reperfusion") occurs frequently and diffuse coronary artery disease is not amenable to revascularization by percutaneous interventions.
- We show that during heart development, the myocardial VEGF-B transgene promotes the formation of endocardium-derived coronary vessels, and after myocardial infarction, VEGF-B provides structural and functional rescue of cardiac tissue.
- The ability of VEGF-B to stimulate *de novo* vessel growth from the ventricles into the myocardium could form a basis for development of new therapeutic strategies for cardiac revascularization after myocardial ischemic injury.

Introduction

Cardiovascular diseases (CVDs) currently form the single most important class of non-communicable diseases and a leading cause of mortality in the western world. CVDs also rank first when scored according to disability-adjusted life years (DALY) criteria[1]. Coronary heart disease is the number one cause of death among the CVDs[2]. The subendocardial region is vulnerable to ischemia and infarction especially in hypertensive patients because it is the most distant target of coronary perfusion, which occurs mainly during the diastolic phase of the cardiac contraction cycle[3]. Currently, the treatment of coronary occlusion is mainly catheter-assisted reperfusion and thrombolysis of the coronary vessels. A timely reperfusion of the myocardium is critical, as adult cardiomyocytes (CMCs) are limited in their capacity to regenerate[4]. However, regardless of the reperfusion, insufficient subendocardial blood flow ("no-flow reperfusion") occurs frequently[5].

Inspired by studies of blood flow in reptilian hearts and human thebesian veins, attempts to induce endogenous angiogenesis were made previously by introducing tissue channels directly to the myocardium from the ventricles. Such trials failed, however, to demonstrate clear functional benefits or improvement of patient survival[6, 7]. The discovery that coronary vessels develop from the sinus venosus[8], endocardium[9] and, to a minor extent, from the epicardium[10, 11], *via* vasculogenesis, angiogenesis, and arteriogenesis[8], has led to the idea that therapeutic reperfusion of the myocardium could be reactivated from these sources in adults. Thus far, this has not succeeded, however[12, 13].

Vascular endothelial growth factor (VEGF), which binds to VEGFR-1 and VEGFR-2, is the major regulator of developmental and adaptive vascular growth in the heart[14]. VEGFR-1, which also binds VEGF-B and placenta growth factor (PlGF), is required for proper embryonic

vasculature development. VEGFR-1 functions mainly as an anti-angiogenic decoy receptor, thus its deletion leads to tissue hypervascularization[15-19]. Despite the fact that VEGF is the strongest angiogenic factor of the three VEGFR-1 ligands, its side-effects, such as promotion of vascular leakage and leukocyte recruitment, have prevented its use as a proangiogenic factor in the treatment of myocardial ischemia[20].

We have earlier shown that transgenic delivery of VEGF-B into the myocardium in mice and rats results in increased coronary vessel size and density and induces a mild non-pathological cardiac hypertrophy[21, 22]. Here, we set out to study the development of a striking coronary vessel phenotype occurring in VEGF-B transgenic (TG) mice and rats. We discovered that the VEGF-B transgene promotes the endocardial contribution to the coronary vasculature, resulting in persistence of ventricle-derived vessels that extend into the subendocardial myocardium. Furthermore, we show that endothelial cells (ECs) in the subendocardial vessels respond to adeno-associated viral vector (AAV)-mediated VEGF-B delivery by proliferation in the adult heart and that, following myocardial infarction (MI), VEGF-B gene transduction can be used to rescue a significant proportion of the myocardial damage resulting from the MI .

Methods

Please refer to the Online-only Data Supplement for an expanded Methods section. Upon reasonable request, the data, analytical methods, and the study materials will be made available to other researchers for repeating the experimental procedures and reproducing the results. Some of the transgenic mouse lines were produced by other researchers and used under the restrictions of material transfer agreements.

Mouse Models

All animal experiments were approved by the animal care committee appointed by the District of Southern Finland. Detailed information on the αMHC-VEGFB, Apln-CreERT2, Npr3-CreERT2, Rosa26-TdTomato$^{lox/STOP/lox}$ transgenic rodent lines, and experimental procedures and treatments used in this study are described in the Online-only Data supplement. The numbers of animals in each experiment are provided in the respective figure legends.

Coronary ligation followed by DiI-perfusion staining

Coronary ligation was performed in five separate experiments with N=5-6 rats per group. Rat hearts were first retrogradely perfused with PBS through the abdominal aorta, followed by ligation of the coronaries at the aortic root when the heart was still pumping. Subsequently, we infused 40 mmol KCl into the cardiac ventricle to stop the cardiac contraction in diastole and then perfused the aorta retrogradely with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI), using a constant pressure infusion pump at a flow rate of 90 ml/hour, followed by 1% paraformaldehyde (PFA), after which the heart was immersed in 4% PFA overnight at room temperature (RT).

Lineage tracing of adult endocardium

Npr3-CreERT^2;R26-tdTomato (6-7 weeks) mice were treated with 3 daily doses of tamoxifen through gavage (2 mg/g weight). One week after last tamoxifen treatment, 2 x 10^{11} AAV-VEGFB or AAV-Ctrl virus particles were injected intraperitoneally (i.p.) and one week thereafter, the LAD was ligated to generate MI. Heart samples were obtained to analyze Npr3-CreER labeled cells (tdTomato+) 4 weeks after the LAD ligation.

Cardiac Echography

Cardiac function was analyzed under isoflurane anesthesia before the start and termination of the study, by using the Vevo 2100 Ultrasound system. From the short-axis M-mode images, left ventricular mass, ejection fraction (EF), fractional shortening (FS), interventricular septum width (diastolic and systolic), left ventricular internal diameter (diastolic and systolic), left ventricular posterior wall width (diastolic and systolic), and left ventricular volume (diastolic and systolic) were calculated. Please also see the **Supplemental Table 1 and 2** for the echocardiographic measurements.

MicroangioCT

Four-month-old αMHC-VEGF-B mice and rats and their wild-type littermates were anesthetized, heparinized, and the thoracic aorta was cannulated in retrograde direction. After washing the blood out with warm heparin (10U/ml)-PBS solution, the mice were perfused with microAngiofil (perfusion speed 0.5 ml/min, for at least 2 ml) and left at room temperature for microAngiofil polymerization for at least 30 minutes, as described earlier[11]. The hearts were covered with wet tissue during that time to prevent sample drying. Thereafter, the hearts were harvested and immersion-fixed in PFA 4% until further processing. At a later time-point, the specimens were scanned using a microCT desktop scanner Skyscan 1272 (Bruker microCT, Belgium) with the following scanning parameters: accelerating voltage 100kV, Cupper filter (0.11mm), isotropic voxel size=$(2.7\mu m)^3$, rotation step 0.1 degrees, 360-degrees scan, frame averaging=ON. The reconstructions represent virtual sections across the obtained dataset. The white color corresponds to the contrast agent located intravascularly and in the ventricles.

Statistical Analysis

The data sets from individual experiments were first tested with Kolmogorov–Smirnov normality tests and were then analyzed with either 2-way ANOVA with a Holm-Sidak post hoc test or a 2-tailed Student's *t* test. $P<0.05$ was considered statistically significant. The data are presented as mean±SEM. GraphPad Prism 7 software was used for the statistical analyses.

Results

VEGF-B transgene promotes growth of cardiac vasculature and mass postnatally. Adult αMHC-VEGF-B mice expressing the VEGF-B transgene in CMCs showed increased coronary vasculature, heart weight/bodyweight ratio and CMC size[21], but no increase in the common pathological cardiac markers (**Fig. 1A,C**). The αMHC-VEGF-B embryos developed normally and were born in Mendelian ratios (**Fig. 1D**). Their coronary vessel areas and heart weight/body weight ratios were found to be significantly increased from postnatal day 7 onwards when compared to those of the wild type (WT) mice (P7) (**Fig. 1E,F**).

Cardiac vessels in VEGF-B transgenic mice and rats are perfused directly from the ventricles

In our earlier study, we found enhanced arterialization of vessels in the hearts of the αMHC-VEGF-B rats[21]. To further study the cardiac vasculature and its development in the TG vs. WT rats, we first performed high-resolution microCT imaging using microAngiofil. This revealed myocardial vessels that originated from the ventricular endocardium in the TG hearts, whereas in the WT hearts, such vessels were not present (**Fig. 2A**). This was interesting, because normally after coronary development, a subendocardial connective tissue layer forms that prevents further vessel growth into the myocardium from the ventricular side[23]. In order to test whether the

vessels originating from the ventricles can deliver blood to the coronary vessels, we ligated, in both the TG and WT rats, all coronaries at the aortic root and then perfused the ventricles retrogradely *via* the aorta with the lipophilic DiI carbocyanine dye that incorporates into EC membranes[24] (**Fig. 2 B-D, Figure I in the Supplement**). Analysis of thick longitudinal sections of the hearts showed prominent DiI staining in the subendocardial vessels and in some vessels across the myocardium in the TG hearts, but not in the WT hearts (**Fig. 2B-D**, **Figure II in the Supplement**). To compare the DiI-positive and negative vessels, we additionally stained the sections with DyLight488-labeled *Lycopersicon esculentum* lectin as an endothelial marker. We found that the DiI-positive vessels in the TG mice were connected to the rest of the coronary vasculature as an integral part of the functional coronary vascular tree (**Fig. 2D, Figure I in the Supplement**). These results indicated that the VEGF-B transgene is capable of promoting development of the subendocardial coronary vasculature that arises *de novo* from the endocardium[9, 25].

AAV-VEGF-B induced gene expression and proliferation in cardiac ECs

In order to study the protective function of VEGF-B delivered to the adult heart, we first tested adeno-associated viral vector (AAV) mediated gene transduction of CMCs. Using AAV-EGFP, expressing the green fluorescent protein and flow cytometry, we first confirmed that the AAV vector transduces the CMCs efficiently. We then also confirmed that the ECs in the AAV-VEGF-B transduced hearts expressed only the endogenous VEGF-B gene (**Figure III in the Supplement**). To analyze how VEGF-B gene delivery modulates the endothelial transcriptome in the adult heart, we performed single-cell RNA sequencing of the cardiac ECs two weeks after AAV-VEGF-B-transduction. For the EC isolation strategy, see **Figure IV in the Supplement.** In the VEGF-B transduced hearts, we found a significant increase of ECs in a cluster representing

highly proliferating ECs (**Fig. 3A-C, Figure V in the Supplement A-B**). The increase in capillary ECs in the S and G2/M phases of the cell cycle is illustrated by the scRNA analysis in **Fig. 3D**. Some of the most prominent changes in gene expression in the coronary and in the endocardial ECs between the AAV-Ctrl and AAV-VEGF-B transduced samples, such as the cell cycle associated transcripts Ccnb2, Birc5 and Top2a, are indicated in the volcano plot analysis (**Fig. 3E-F**). This analysis also confirmed that *Pecam1*, which normally is expressed less in endocardial cells than in coronary vessel ECs, and *Nrg1*, which is necessary for myocardial trabeculation during development[26], were upregulated in endocardial ECs from the VEGF-B transduced hearts (**Fig. 3F**). Furthermore, transcripts encoding the endocardium-specific markers *Cdh11* or *Npr3* were higher in the VEGF-B transduced hearts (**Fig. 3F**). A shift towards activated and proliferating ECs was also apparent in the Velocyto analysis of the data[27] (**Figure V in the Supplement C).**

VEGF-B activates ECs throughout the myocardium, but induces proliferation mainly in subendocardial ECs

After intraperitoneal (i.p.) injection of the DNA-integrating proliferation marker 5-ethynyl-2'-deoxyuridine (EdU), we found about 3-fold more EdU^+ EC nuclei in the subendocardium than in the subepicardium of the AAV-VEGF-B-transduced hearts, whereas the AAV-Ctrl transduced myocardium showed essentially no EdU-labeling (**Fig. 4A-B**). Interestingly, EdU+ nuclei were also observed in the endocardium of the AAV-VEGF-B hearts (**Fig. 4B**). A similar increase was evident in the Ki67+ EC nuclei two weeks after AAV-VEGF-B transduction, but no longer after four months (**Figure VI in the Supplement**). AAV-mediated transduction of the other VEGFR-1 ligand, PlGF, also induced proliferation of subendocardial ECs (**Figure VII in the Supplement**).

AAV-VEGF-B transduction resulted in an increase of cells in the activated EC cluster 5, characterized by their increased expression of e.g. apelin (Apln)[28] (**Figure V in the Supplement A**). To localize these cells, we injected AAV-VEGF-B or AAV-Ctrl into AplnCreERT2;Td-tomato reporter mice[28]. Two weeks after gene transfer, followed by tamoxifen administration, the VEGF-B transduced cardiac sections showed two- to three-fold more Apelin$^+$ ECs than did the Ctrl samples in both the subepicardium and the subendocardium (**Figure VIII in the Supplement**). These results indicate that, whereas VEGF-B leads to increased EC proliferation especially in the subendocardial myocardium, the coronary endothelial activation, evident as Apln+ cells, occurred more homogenously in the various parts of the myocardium.

Cell lineage tracing reveals endocardial contribution to coronary vessels after MI only in VEGF-B expressing hearts

To ask whether the vessels that we observed arise from the endocardium and whether VEGF-B can induce endocardial transdifferentiation into coronary vascular endothelium after MI, we performed genetic lineage tracing of endocardial cells in the adult heart and analyzed their cell fate after MI. We used the Npr3-CreER allele for lineage tracing of the adult endocardium[12]. We injected tamoxifen into Npr3-CreER;R26-Td-Tomato mice, treated the mice with AAV-VEGF-B or AAV-Ctrl, and then ligated the left descending coronary artery to induce MI. We then analyzed cardiac sections from the mice four weeks after LAD ligation (**Fig. 5A**). In mice without MI, immunostaining for Td-Tomato (red) and the vascular EC marker FABP4 (green) showed Td-Tomato fluorescence only in the endocardial cells, but not in the FABP4+ vascular ECs (**Fig. 5B,C**). After MI or a sham operation, almost all endocardial TdTomato$^+$ cells remained in the innermost layer of the myocardial wall in the AAV-Ctrl group. In the VEGF-B transduced sham operated hearts, only a few TdTomato$^+$ cells contributed to FABP4$^+$ vascular

ECs in the myocardium, whereas after MI, a robust increase in the endocardium-derived TdTomato$^+$ ECs was observed in the subendocardial vessels of the AAV-VEGF-B transduced hearts (**Fig. 5D**). Statistical analysis showed a significant increase in the vascular endothelial contribution from the endocardium in the AAV-VEGF-B vs. AAV-Ctrl group, but only after MI (**Fig. 5E**). Furthermore, lectin perfusion experiments indicated that at least some of the endocardium-derived vessels were functional (**Fig. 5G, Figure IX in the Supplement**). Taken together, the genetic lineage tracing results demonstrated that VEGF-B gene delivery before LAD ligation is capable of enhancing the endocardial contribution to *de novo* coronary vessels after injury (**Fig. 5F**).

VEGF-B gene delivery before or immediately after transient ischemia reduces cardiac area at risk and scar tissue after MI

We then analyzed the functional importance of the timing of VEGF-B gene delivery in relation to LAD ligation. Histological and immunohistochemical staining of the myocardial scar tissue four weeks after the MI showed smaller scars in the AAV-VEGF-B treated mice than in the AAV-Ctrl treated mice (**Fig. 6A-B**). Due to the difference in the scar size, we also decided to assess the unperfused "area at risk" 24 hours after MI, using Evans blue and triphenyltetrazolium chloride (TTC) staining, as described earlier[29]. Similarly to the Masson Trichrome staining, the scar area was significantly smaller in the AAV-VEGF-B treated hearts, and the perfused area was significantly larger, whereas the area-at-risk was the same. In particular, the subendocardial area around the ventricles was perfused in the AAV-VEGF-B treated hearts but not in the AAV-Ctrl treated hearts (**Figure X in the Supplement**). This confirmed that the VEGF-B-induced vessels alleviated myocardial ischemia.

VEGF-B increases the number of proliferating cardiac ECs after MI

To understand the transcriptomic changes that VEGF-B induces in ischemic myocardium, we performed scRNA sequencing of ECs from the AAV-VEGF-B and AAV-Ctrl hearts seven days after MI (**Figure XI and XII in the Supplement**). The most significant differences between these ECs were detected in the proliferating cell cluster, representing the cell cycle S phase (cluster 12) and G2M phase (clusters 10 and 15), which were significantly larger in samples from the VEGF-B transduced hearts (**Figure XI in the Supplement**). Interestingly, *Cxcl12,* which binds to CXCR4, was upregulated in several clusters only in the infarcted VEGF-B transduced heart. This may be significant, because a recent study showed that arterial ECs express CXCR4, and following injury, capillary ECs increase CXCL12 expression, which leads to arterial reassembly and collateralization[30].

VEGF-B gene deletion and gene transfer on heart function after MI

We next analyzed whether the endogenous VEGF-B gene affects the outcome of LAD ligation. Our earlier studies indicated that the expanded coronary vasculature in the VEGF-B TG rats protects against MI upon LAD ligation[22]. However, we found that neither cardiac ejection fraction (EF) nor fractional shortening (FS) was altered by VEGF-B gene deletion (**Table I in the Supplement**). We then tested the functional benefit of AAV-VEGF-B transduction of the adult mouse heart six or three days before MI, or immediately following MI. We found that AAV-VEGF-B transduction, measured two weeks after MI, improved EF and FS (**Fig. 6C, Table II in the Supplement**). An improved EF was also evident in AAV-VEGF-B transduced hearts two weeks after a 45-minute ischemia-reperfusion treatment (**Figure XIII in the Supplement**). Consistent with the histological findings, after the MI, AAV-VEGF-B expressing

mice also retained significantly thicker myocardial walls than did the AAV-Ctrl mice (**Table II in the Supplement)**.

Discussion

Here we present the striking observation that myocardial expression of the VEGF-B transgene leads to persistence of cardiac vessels derived from the endocardium that perfuse the subendocardial myocardium. Furthermore, in adult mice, the delivery of a VEGF-B transgene led to proliferation of coronary ECs particularly in the subendocardial myocardium. Myocardial VEGF-B gene transduction, occurring prior to or immediately after LAD ligation, promoted transdifferentiation of endocardial cells into vascular ECs and their participation in the growth of the coronary vasculature, as well as improved cardiac function after MI. These results suggest that during the myocardial remodeling process after MI, VEGF-B can reactivate the endocardial contribution to coronary vessel formation. This means that, in ischemic heart disease, VEGF-B has the potential of augmenting the development of endocardium-derived cardiac neovascularization.

During mouse development, endogenous VEGF-B expression starts at E10.5 in the CMCs of the right ventricle and in the interventricular septum, whereas the atria express much less VEGF-B[31]. During postnatal angiogenesis associated with the massive growth of the myocardial wall, VEGF-B expression switches to the LV wall, becoming downregulated after postnatal cardiac remodeling to levels observed in the adult heart[31]. In the αMHC-VEGF-B mice, the transgene-encoded VEGF-B is secreted by CMCs, which are also the major source of endogenous VEGF-B in the WT hearts[32]. Thus, although the endogenous VEGF-B expression is not essential for cardiac development or function[31], an excess of VEGF-B provided by the

transgene is able to further expand the coronary vasculature during postnatal growth when endogenous VEGF expression is elevated. Importantly, after postnatal development, both the vasculature and the cardiomyocyte size were stabilized in the transgenic hearts, thus the relative increase in the vasculature and in the CMC cross-sectional area in 2-month-old and 2-year-old rats are similar[22].

In the developing heart, the sinus venosus (SV)[8] and the endocardium provide the major sources of coronary vessels[9, 25], which grow in three major waves to support the expanding myocardium during gestation and neonatally[33]. The majority of the coronary vessels in the ventricular free wall originate from the SV endocardium, whereas interventricular septal vessels develop from the ventricular endocardium[10]. In the adult heart, the extent of endocardial contribution to the formation of coronary ECs after an injury, such as MI, is minimal, although it may depend on the extent of hypoxic damage[34, 35]. The increased subendocardial vascularization that we found in the hearts expressing the VEGF-B transgene is important, as the subendocardial myocardium is particularly vulnerable to myocardial infarction[36]. Research in the field has suggested that a lower blood supply to the subendocardium and higher compliance of the subendocardial vessels leads, in cardiac ischemia, to the subendocardial vulnerability[3].

During the cardiac development of vertebrates, the trabecular myocardium undergoes compaction regulated by paracrine signals between the endocardium and the myocardium[13]. The Notch and Neuregulin pathways are essential for this process, which starts before the heart tube assembly at E8.0[26]. In mouse cardiac development, the endocardium-to-vessel transition occurs during trabecular compaction morphogenesis[26, 37]. In this process, the endocardial cells that are trapped between the compacting myocardial trabeculae undergo a phenotypic transition to vascular ECs, and they also generate fibroblasts and smooth muscle cells[23]. The endocardium

thus forms the origin of subendocardial vessels that anastomose with coronary vessels derived from the SV and become perfused. Interestingly, this process of endocardial "touchdown" formation, in which endocardial cell ridges encapsulate the trabecular myocardium and start to form the initial trabecular architectural units, corresponds to the angiogenic sprouting in other vascular beds[38].

Vessels that emerge from the cardiac ventricles occur in mammals and in lower species, in which they are believed, on the one hand, to provide a myocardial drainage route, and on the other hand, to nourish the myocardium with oxygenated blood[39]. Such venous connections are called "Thebesian veins", and arterialized vessels called vessels of Wearn[40, 41]. In humans, the number of such vessels is normally between 0 and 10, and in unchallenged conditions their role thus seems insignificant[39]. In non-mammalian species, however, these vessels play a role in perfusing the intertrabecular region, with their anastomoses to the coronary vasculature[40].

Even though adult endocardial cells contribute only minimally to cardiac ECs after injury, endocardial cells that become entrapped in the myocardium during post-ischemic remodeling may contribute to the vessel growth in the damaged myocardial tissue[12]. Thus, endocardial tissue remodeling could offer a source for cardiac neovascularization in pathological conditions. This idea is consistent with our present results, showing that in the adult heart, the tissue remodeling after MI was required for the VEGF-B-induced endocardial-to-vascular endothelial contribution in the regenerating vessels. However, whereas the endogenous program makes none at all or only a minimal contribution to the coronary circulation[42], our results indicate that VEGF-B is the first factor found that can induce both vessel growth and endocardial cell delamination from the endocardium and migration into the ischemic myocardium, where

endocardial ECs transdifferentiate into vascular ECs and participate in *de novo* coronary vessel formation.

Because the reperfusion of the subendocardial region of the heart is a major problem, we examined AAV-gene therapy as a safe and feasible method to deliver VEGF-B for the stimulation of cardiac subendocardial vessel growth. Upon VEGF-B transduction, we found a clear increase in proliferating ECs, located mostly in the subendocardial region. This is most likely due to angiogenesis stimulated indirectly by VEGF-B *via* VEGF-VEGFR-2 signaling, which is in agreement with the increased vasculature density previously observed in AAV-VEGF-B-transduced hearts[21]. Moreover, VEGF-B167 protein, delivered *via* a slow release minipump has enhanced revascularization of the myocardial border zone after infarction caused by LAD-ligation[43]. A cardiomyocyte-specific VEGF-B transgene was also associated with reprogramming of CMC metabolism and preservation of mitochondrial complex I function upon ischemia-reperfusion[22]. In dogs subjected to tachypacing-induced cardiomyopathy, VEGF-B167 markedly preserved diastolic and contractile function and attenuated ventricular chamber remodeling, halting the progression from compensated to decompensated heart failure[44]. These studies suggest that VEGF-B has therapeutic effects in regard to myocardial ischemia and heart failure.

VEGF-B expression was reported to decline in heart failure patients, where circulating VEGF-B levels are inversely correlated with ongoing left ventricular remodeling[45, 46]. We considered that myocardial delivery of VEGF-B could be therapeutic in cardiac remodeling after acute ischemia/injury, when the subendocardium reverts transiently to its former hypertrabeculated state, which may facilitate endocardium-derived neovascularization[35, 47]. We furthermore considered that such effect of VEGF-B could be enhanced after MI, because

VEGFR-2, which transduces the VEGF-B-mediated angiogenic signals, is upregulated after MI also in endocardial flower-shaped structures that contain proliferating ECs[47]. In agreement with our hypothesis, AAV-VEGF-B transduction six days before the MI prevented the decrease of cardiac EF. Surprisingly, a protective effect on the cardiac function was observed even when AAV-VEGF-B was injected three days before, or even just after the LAD ligation, indicating for the first time that the coronary endothelial growth promoted by VEGF-B gene delivery is beneficial during acute cardiac injury. These results suggest that VEGF-B gene delivery could be useful in inoperable acute coronary syndrome patients or in combination with balloon angioplasty to overcome the often-occurring no-reflow phenomenon.

VEGF-B thus fulfills many of the desired features for a vascular perfusion-enhancing agent. It has been shown to protect from microvascular defects generated during cardiac stress, an important aspect for avoidance of the no-flow phenomenon and successful reperfusion[22]. It also promotes vessel arterialization[21], which is crucial for pressurized blood delivery into the cardiac muscle. Furthermore, a clear advantage of VEGF-B in comparison with other angiogenic factors is that it cannot be overdosed. This is because an excess of VEGF-B works merely by binding to the VEGFR-1 decoy receptor, thereby displacing the endogenous VEGF for induction of angiogenesis through the VEGFR-2-signaling pathway, which in turn promotes mild CMC growth through paracrine EC-CMC crosstalk[48]. It is important to note, that the therapeutic dosing window is much smaller for VEGF-A than for VEGF-B, because VEGF-A is poorly angiogenic in the heart[49], and its side effects, such as vascular leakage and inflammation are greater, which thus far has compromised its use in the treatment of cardiovascular ischemic conditions [21, 22, 50].

In conclusion, we present a novel VEGF-B function in the endocardial transdifferentiation to vascular ECs, associated with the development of endocardium-derived

blood vessels in the heart. Our study shows that VEGF-B is capable of reactivating an organ-specific developmental growth program that is distinct from angiogenic sprouting in other developing vascular beds, thus overcoming the insufficiency of intrinsic developmental mechanisms that drive new vessel formation in the injured heart (**Figure XIV in the Supplement**). Studies of the endocardial transcriptome and possible progenitor-like EC clusters are required to find out how VEGF-B alters the endocardial cells during development and repair of the coronary vessels and how it can be further boosted for an improved functional and eventually therapeutic benefit. This is a translationally and clinically relevant research area, which could provide a new possibility for the development of better myocardial perfusion to the injured heart directly from the cardiac ventricles.

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M.R., R.K, and K.A. designed, and M.R. I.S. J.P. and K.A.H. performed the mouse and rat experiments and analyzed the data. W.Y. and J.T. performed and B.Z. designed and interpreted the AAV-VEGF-B endocardial lineage tracing experiments. L.H. and Y.S. analyzed and C.B. interpreted bioinformatic data. R.H. and V.D. designed and O.Z.K. performed the microCT-imaging experiment. M.R. R.K., B.Z. and K.A. wrote the manuscript. All authors have seen, commented and accepted the manuscript.

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Disclosures

None.

Supplemental Materials

Expanded Methods

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Figure Legends

Figure 1. Cardiac phenotype and its development in the αMHC-VEGF-B TG mice. (A) Macroscopic images of the hearts and ANP and BNP RNA levels at 4 months **(B)** IF staining of cardiac sections for myocytes (dystrophin-2) and blood vessels (CD31) **(C)** Quantification of cardiomyocyte size (μm²) and blood vessel area. **(D)** Percentage of TG embryos, pups, and adults at the indicated time points **(E)** Blood vessel area fraction. **(F)** Heart weight normalized to body weight (mg/g) at the indicated embryonic and postnatal days. N=8 per group, mean ± SEM, **p<0.01 & *p<0.05. Scale bar= 300 μm.

Figure 2. The ventricle-derived blood vessels in αMHC-VEGF-B TG rats are lumenized and connect to coronary blood vessels. (A) MicroCT imaging after retrograde perfusion of the ventricles with microAngiofil contrast agent. Arrows indicate blood vessels connected to the ventricle. **(B)** Schematic illustration of the ligation of coronary vessels and quantifications of DiI perfused vasculature fraction. **(C)** DiI staining after coronary ligation and retrograde ventricular perfusion of the WT and TG hearts. **(D)** Immunofluorescent images of DiI and Lycopersicon esculentum lectin staining of the subendocardium in the septal region. N=6 rats per group, Scale bars: **(C)** 1 cm **(D)** 100 μm, LCA=left coronary artery, RCA=right coronary artery.

Figure 3. Increased expression of transcripts associated with EC activation and proliferation in AAV-VEGF-B transduced hearts. (A) UMAP clustering of cardiac ECs from AAV-VEGF-B and AAV-Ctrl transduced mice. Cluster 8, which contains hemoglobin transcripts was not present consistently in the samples and was considered a contaminant. (**B)** Relative

proportions of cells in the major EC clusters between the AAV-VEGF-B and AAV-Ctrl transduced samples. Note the greater number of proliferating ECs in the VEGF-B transduced sample. (**C)** UMAP plots showing the cell cycle phase of each single cell in both samples. (**D)** S and G2M scores in the samples. (**E-F**) Volcano plots of genes differentially expressed in ECs (**E**) and endocardial cells (**F**) between the samples. The threshold is adjusted to a logFC > 0.25 and FDR < 0.05.

Figure 4. AAV-VEGF-B promotes EC proliferation predominantly in the subendocardial myocardium. (A) Schematic outline of the EdU+Apelin lineage tracing experiment. **(B)** Representative images and quantification of subendocardial and subepicardial %EdU+ nuclei/field (N=6 mice/group). Yellow arrowheads point out the EdU+/CD31+ nuclei. Mean +/- SEM, ***p<0.0005, *p<0.05

Figure 5. VEGF-B promotes endocardial contribution to vessel endothelium in the adult heart after MI. (A) Schematic figure showing the experimental strategy. **(B)** Immunostaining of Td-Tomato and FABP4 in sections for Npr3-CreER;R26-Td-Tomato TG hearts. **(C)** Quantification of the percentage of Td-Tomato+ cells among endocardial cells and FABP4+ coronary endothelial cells. **(D)** Td-Tomato and FABP4 immunostaining of heart sections after MI or sham operation. Note that a subset of endocardial cells contributes to FABP4+ vascular endothelial cells (VECs) after AAV-VEGF-B treatment (arrowheads). **(E)** Quantification of the percentage of Td-Tomato+, FABP4+ cells in relation to the infarcted area. **(F)** Cartoon image illustrating increased endocardial contribution after MI. **(G)** Immunostaining for tdTomato and

VE-cadherin on hearts collected 14 days after MI. Mice were perfused with BS lectin before they were collected. Mean ± SEM, ***p<0.001, **p<0.01. Scale bar: 100 μm. N=5 mice per group.

Figure 6. VEGF-B gene therapy decreases myocardial scar and improves cardiac ejection fraction after MI. (A) Masson Trichrome staining of the hearts and **(B)** quantification showing the scar dimensions when AAV was given 6 days before the MI. **(C)** cardiac function presented as ejection fraction (EF) and left ventricular mass (LV mass) measured from the echocardiography two weeks after MI, in 3 separate experiments, in which AAV was given either six or three days or right after the LAD ligation (N=6 mice for shams and N=10 mice for MI per group). Mean+/-SEM. *p<0,05, ** p<0,005.